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## **Ethanol Origin in Postmortem Urine: An LC/MS Determination of Serotonin Metabolites**

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16. Abstract  Specimens from fatal aviation accident victims are submitted to the FAA Civil Aerospace Medical Institute for toxicological analysis. During toxicological evaluations, ethanol analysis is performed on all cases. Care must be taken when interpreting a positive ethanol result due to the potential for postmortem ethanol formation. Several indicators of postmortem ethanol formation exist; however, none are completely reliable. The consumption of ethanol has been shown to alter the concentration of two major serotonin metabolites, 5-hydroxytryptophol (5-HTOL) and 5-hydroxyindole-3-acetic acid (5-HIAA). While the 5-HTOL/5-HIAA ratio is normally very low, previous studies using live subjects have demonstrated that the urinary 5-HTOL/5-HIAA ratio is significantly elevated for 11-19 hours after acute ethanol ingestion. We investigated the 5-HTOL/5-HIAA ratio as a potential indicator of ethanol origin in postmortem urine samples. We developed and validated a method for the simultaneous determination of 5-HTOL and 5-HIAA in forensic urine samples using a simple liquid/liquid extraction and LC/MS/MS and LC/MS/MS/MS. A typical recovery of approximately 80% was achieved for both compounds. The LC/MS method proved highly selective and sensitive, having an LOD of 0.1 ng/mL for both compounds. The accuracy and precision was also very good. Utilizing our LC/MS method, we examined the 5-HTOL/5-HIAA ratio in 21 ethanol-negative and 23 true ethanol-positive postmortem urine specimens. We found that all ethanol-negative specimens had 5-HTOL/5-HIAA ratios below 15 pmol/nmol, a previously established antemortem urine cutoff for recent ethanol ingestion. All ethanol-positive urine samples had 5-HTOL/5-HIAA ratios above 15 pmol/nmol. These results validated the antemortem cutoff for use with postmortem urine specimens. This method was then applied to cases where postmortem ethanol formation was suspected.					
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## ETHANOL ORIGIN IN POSTMORTEM URINE: AN LC/MS DETERMINATION OF SEROTONIN METABOLITES

### INTRODUCTION

The Federal Aviation Administration's (FAA's) Civil Aerospace Medical Institute (CAMI) is responsible, under Department of Transportation (DOT) orders 8020.11A and 1100.2C, to "conduct toxicologic analysis on specimens from ... aircraft accident fatalities" and "investigate ... general aviation and air carrier accidents and search for biomedical and clinical causes of the accidents, including evidence of ... chemical (use)." Therefore, following an aviation accident, samples are collected at autopsy and sent to CAMI's Toxicology and Accident Research Laboratory, where toxicological analysis is conducted on various postmortem fluids and tissues.

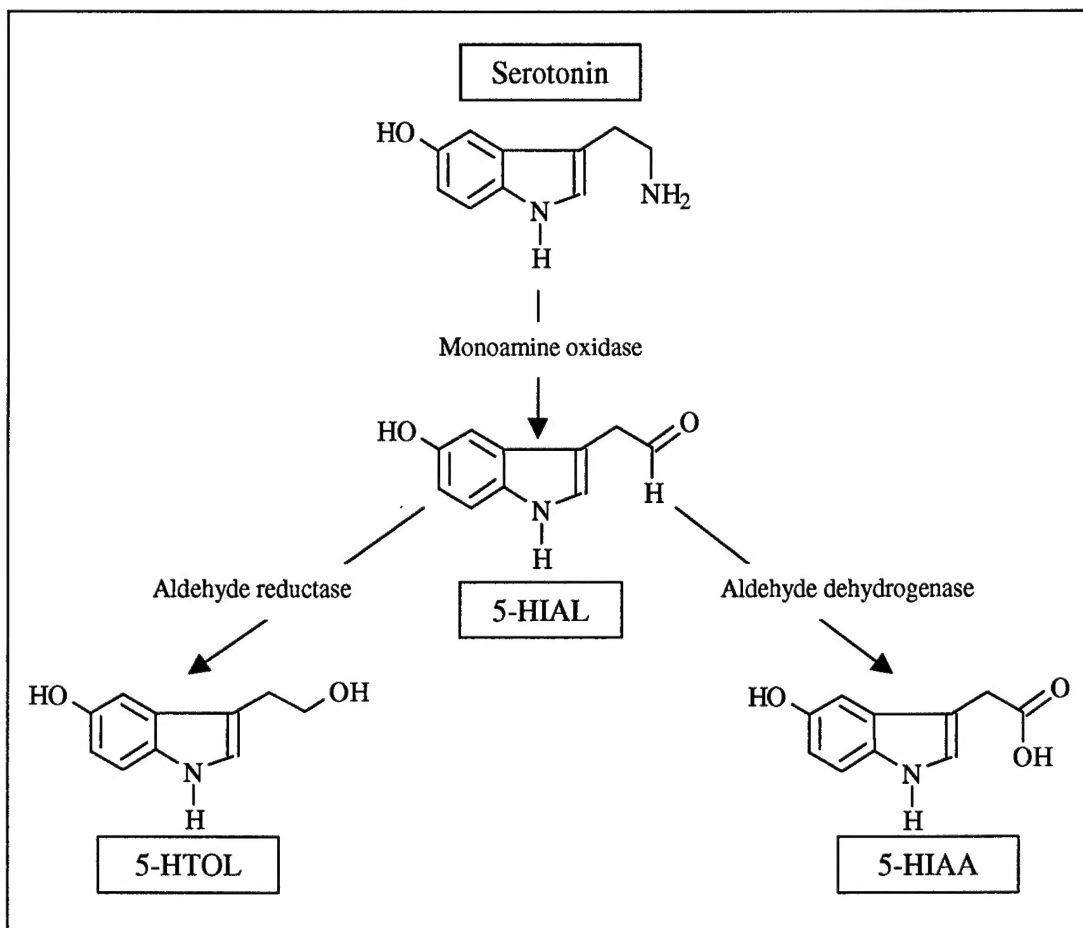
Ethanol analysis is one of the most common tests performed on forensic specimens. The presence of ethanol in fatal aircraft accident victims constitutes an important part of aircraft accident investigations and litigation. An ethanol positive also invites legal scrutiny as to its validity. With the accuracy of today's analytical techniques, there should be no doubt concerning the quantitative determination of ethanol found in a biological specimen. However, the origin of the ethanol found in a specimen is another, and highly pertinent, variable to consider when interpreting ethanol results.

The microbial formation of ethanol in postmortem specimens is the most significant problem encountered when evaluating ethanol results. The first report dealing with postmortem formation of ethanol in corpses appeared in 1936.<sup>1</sup> But while a postmortem increase in ethanol was observed, the precise source of the increase was unknown. Today, it is known that many different microbes may be responsible for postmortem formation of ethanol. Investigations have been performed to identify the particular species of bacteria, yeast and/or fungi primarily responsible for ethanol production and the mechanism by which ethanol is formed.<sup>2,3,4,5</sup> *Candida albicans* has been identified as the microbe most often responsible for postmortem production of ethanol in humans.<sup>6,7</sup> This species of yeast is commonly found in humans *in vivo*. Although located ubiquitously throughout the body, the highest concentrations are typically in the mouth and on the skin.<sup>8</sup> Glucose is the most prevalent substrate in the human body used by these microbes to form ethanol.<sup>9</sup> Other endogenous compounds can also be utilized as substrates including, but not limited to, lactate, mannitol, galactose, maltose, sucrose and lactose.<sup>9,10,11,12</sup>

When working with forensic specimens, postmortem ethanol formation is generally avoided by storage at appropriate temperatures and the addition of preservatives such as sodium fluoride.<sup>13</sup> These precautions, however, do not rule out the possibility of ethanol formation prior to sample collection and preservation.<sup>14</sup> Furthermore, studies have demonstrated that if a specimen's storage temperature is not carefully controlled, the preservative sodium fluoride may become ineffective in blocking postmortem formation of ethanol.<sup>15,16</sup> Although several methods are available to help address the postmortem ethanol issue, in many cases, the source of ethanol remains elusive.

Recently, exploitation of the metabolism of serotonin as a biological marker for ethanol consumption has begun to gain interest in the field of forensic science. Serotonin (5-hydroxytryptamine, 5-HT) is an indoleamine commonly found in nature.<sup>17</sup> In humans, 5-HT is found throughout the body, with substantial concentrations found in the gastrointestinal tract and blood platelets.<sup>18</sup> The metabolism of 5-HT initially involves oxidative deamination to form the intermediate aldehyde, 5-hydroxyindole-3-acetaldehyde (5-HIAL). This intermediate can undergo either oxidation or reduction as shown in Figure 1. Oxidation of the aldehyde, catalyzed by aldehyde dehydrogenase, leads to formation of 5-hydroxyindole-3-acetic acid (5-HIAA).<sup>19</sup> Reduction, catalyzed by aldehyde reductase, leads to formation of 5-hydroxytryptophol (5-HTOL).<sup>20</sup> Under normal circumstances 5-HIAA is the predominant metabolite of 5-HT.<sup>18</sup> However, ethanol consumption has been shown to significantly alter the normal metabolism of serotonin.

An increase in 5-HTOL concentration following ethanol consumption was first reported in 1967.<sup>21</sup> Since that time it has been clearly demonstrated that the consumption of ethanol shifts serotonin metabolism to favor formation of 5-HTOL and reduces the formation of 5-HIAA.<sup>20,22,23,24</sup> Two primary factors contribute to this increase in the 5-HTOL/5-HIAA ratio. The first factor involves the oxidation of ethanol into acetaldehyde. As acetaldehyde is produced, it is rapidly oxidized by aldehyde dehydrogenase. The oxidation of acetaldehyde results in competitive inhibition of aldehyde dehydrogenase and prevents its contribution to 5-HIAA formation.<sup>25</sup> The second factor capable of increasing the 5-HTOL/5-HIAA ratio is the higher than normal concentrations of the reduced form of nicotinamide adenine dinucleotide (NADH), resulting



**Figure 1.** The metabolism of serotonin.

as a byproduct from the oxidation of both ethanol and acetaldehyde.<sup>26</sup> The oxidation of these two compounds consumes  $\text{NAD}^+$ , a necessary cofactor for aldehyde dehydrogenase activity, and creates an excess of  $\text{NADH}$ , a cofactor for aldehyde reductase, thereby promoting the formation of 5-HTOL. These two factors combine to dramatically increase the 5-HTOL/5-HIAA ratio following ethanol ingestion.<sup>25</sup> The 5-HTOL/5-HIAA ratio remains elevated for hours after ethanol has been eliminated from the body.<sup>23</sup> As such, the 5-HTOL/5-HIAA ratio has been applied, in a limited manner, to ethanol cessation monitoring programs as a marker of recent ethanol ingestion in urine specimens. The 5-HTOL/5-HIAA ratio has also been briefly examined in postmortem urine samples. This ratio may serve as a powerful tool in forensic science for differentiating between the postmortem formation and ingestion of ethanol.

Historically, levels of 5-HTOL and 5-HIAA have been examined using two considerably unrelated analytical techniques. 5-HIAA concentrations are easily measured at sub-nanomolar levels using liquid chromatography with electrochemical detection (LC/EC).<sup>27, 28</sup> 5-HTOL may also be detected with LC/EC, but the detection limits are

typically insufficient to monitor this compound in most pertinent biological specimens. For this reason, 5-HTOL has typically been analyzed using gas chromatography with mass spectrometric detection (GC/MS).<sup>29</sup> The employment of two different analytical techniques to obtain a 5-HTOL/5-HIAA ratio in a specimen obviously increases the probability of substantial errors. To date this has effectively prevented the widespread use of this ratio as a marker for ethanol ingestion. In this study we describe a method for the rapid and simultaneous determination of 5-HTOL and 5-HIAA using liquid chromatography with mass spectrometric detection (LC/MS). Following validation of the method, we investigated the 5-HTOL/5-HIAA ratios in both ethanol-positive and ethanol-negative postmortem urine samples.

## MATERIALS AND METHODS

### Chemicals and Solutions

All aqueous solutions were prepared using double deionized water (DDW), which was obtained using a Milli-QT<sup>plus</sup> Ultra-Pure Reagent Water System (Millipore®, Continental Water Systems, El Paso, TX). All

chemicals were purchased in the highest possible purity and used without any further purification. Sodium chloride, sodium acetate,  $\beta$ -glucuronidase, 5-hydroxytryptophol, 5-hydroxyindole-3-acetic acid and 5-methoxy-2-methyl-3-indoleacetic acid were purchased from Sigma Chemical Company (St. Louis, MO). Methanol, acetonitrile, ammonium hydroxide, hydrochloric acid, ethyl acetate and nitric acid were purchased from Fisher Scientific (Pittsburgh, PA). Formic acid (97%) was purchased from ICN (ICN Biomedicals, Inc., Irvine, CA). N,O-bis[trimethylsilyl]trifluoroacetamide with 1% trimethylchlorosilane (BSTFA/1%-TMCS) was purchased from Pierce (Pierce Chemicals, Rockford, IL).

Stock solutions of 5-HTOL and 5-HIAA were prepared at 1.00 mg/mL (5.64 mM 5-HTOL; 5.23 mM 5-HIAA) in methanol. 5-Methoxy-2-methyl-3-indoleacetic acid (5-MMIA) was used as the internal standard for these experiments and was prepared at a concentration of 100  $\mu$ g/mL (0.450 mM) in methanol. These compounds are light sensitive, so care was taken to use volumetric flasks wrapped in aluminum foil to prevent photodegradation. Once prepared, the solutions were transferred to 20 mL amber glass bottles, capped and placed in the freezer for storage at  $-20^{\circ}\text{C}$ . These solutions were used for 1 month and then discarded.  $\beta$ -glucuronidase solution was prepared by adding 2.5 mL 0.1 mM sodium acetate buffer pH 5.00 to 250,000 units of enzyme, yielding a final concentration of 100,000 units/mL. This solution was stored in the freezer at  $-20^{\circ}\text{C}$  and expired after 1 month.

The aqueous portion of the HPLC buffer was 50.0 mM formic acid adjusted to pH 5.00 with conc. ammonium hydroxide. Aqueous buffer and acetonitrile were mixed in a 98:2 ratio, respectively, to help prevent the growth of microbes. The mixture was filtered through a vacuum filtering apparatus that incorporated a 0.45  $\mu$ m GH polypro 47 mm hydrophilic, polypropylene membrane filter obtained from Pall Gelman laboratory (Pall Corp., East Hills, NY). The organic portion of the mobile phase was HPLC grade methanol, which was filtered prior to use through a vacuum filter apparatus that incorporated the same type of membrane filter as above.

### Instrumentation

Analyte separation was achieved using a Hewlett Packard 1100 HPLC (Hewlett Packard Co., Wilmington, DE) equipped with a Security Guard<sup>TM</sup> C-8 guard column (4.0 mm x 3.0 mm i.d., 3  $\mu$ m particles) from Phenomenex<sup>®</sup> (Torrance, CA), followed immediately by a Supelcosil<sup>TM</sup> LC-18 (150 mm x 4.6 mm i.d., 3  $\mu$ m particles) analytical column from Supelco (Supelco/Sigma-Aldrich, Bellefonte, PA). Samples were injected using a Hewlett Packard G1313A autosampler (Hewlett Packard Co., Wilmington, DE). Identification and quantitation were

accomplished using a Finnigan model LCQ atmospheric pressure chemical ionization (APCI) ion trap mass spectrometer (ThermoFinnigan Corp., San Jose, CA). Control of the HPLC system, integration of the chromatographic peaks and communication with the mass spectrometer were accomplished using a Gateway 2000 E-4600-SE personal computer using Xcalibur<sup>TM</sup> LC/MS software (ThermoFinnigan Corp., San Jose, CA).

### LC/MS/MS and LC/MS/MS/MS Method

Working with standards of 5-HIAA and 5-HTOL, we began the initial investigation by looking into the response received from these 2 compounds when injected directly into the LCQ. As described in detail below, 5-HTOL and 5-HIAA were derivatized with TMS to form 5-HTOL-TMS and 5-HIAA-TMS derivatives. Initial ionization evaluation of these derivatives indicated that positive chemical ionization (PCI), creating the  $[\text{M}+\text{H}]^+$  ions, was more effective than negative chemical ionization (NCI), which formed the  $[\text{M}-\text{H}]^-$  ions. APCI-PCI-MS conditions were optimized by infusing the desired compound at approximately 10  $\mu$ g/mL into the LCQ at 1 mL/min. Tuning the MS for the desired ions was accomplished using the autotune feature of the Xcalibur<sup>TM</sup> software. As a result of preliminary APCI-PCI-MS investigations, each sample analysis was subsequently split into 3 unique data collection segments.

The operating conditions for segment 1, which analyzed for 5-MMIA, were as follows: APCI capillary temperature,  $150^{\circ}\text{C}$ ; APCI vaporizer temperature,  $450^{\circ}\text{C}$ ; source voltage, 10.0 kV; source current, 5.0  $\mu$ A; capillary voltage, 8.0 V; tube lens offset, 25.0 V; octapole 1 offset, -1.75 V; octapole 2 offset, -6.5 V; interoctapole lens voltage, -16.0 V; ion trap DC offset, -10 V; multiplier voltage, 0.0 V; micro-scan injection time, 200 msec. Additionally, segment 1 was split into 2 separate scan events. Scan event 1 collected the  $[\text{M}+\text{H}]^+$  parent ion at  $m/z$  220.1, and scan event 2 performed MS/MS on this parent ion using a collision energy of 42% and collected the daughter ion at  $m/z$  174.1.

The operating conditions for segment 2, which analyzed for 5-HTOL, were as follows: APCI capillary temperature,  $150^{\circ}\text{C}$ ; APCI vaporizer temperature,  $450^{\circ}\text{C}$ ; source voltage, 10.0 kV; source current, 5.0  $\mu$ A; capillary voltage, 17.0 V; tube lens offset, 25.0 V; octapole 1 offset, -3.25 V; octapole 2 offset, -7.0 V; interoctapole lens voltage, -16.0 V; ion trap DC offset, -10 V; multiplier voltage, 0.0 V; micro-scan injection time, 200 msec. Additionally, segment 2 was split into 3 separate scan events. Scan event 1 collected the  $[\text{M}+\text{H}]^+$  parent ion at  $m/z$  250.1. Scan event 2 performed MS/MS on this parent ion, using a collision energy of 38%, and collected the daughter ion at  $m/z$  232.1. Scan event 3 performed



MS/MS/MS on the daughter ion at  $m/z$  232.1, using a collision energy of 48%, and collected the granddaughter ion at  $m/z$  216.1.

The operating conditions for segment 3, which analyzed for 5-HIAA, were as follows: APCI capillary temperature, 150°C; APCI vaporizer temperature, 450°C; source voltage, 10.0 kV; source current, 5.0  $\mu$ A; capillary voltage, 3.0 V; tube lens offset, 10.0 V; octapole 1 offset, -4.25 V; octapole 2 offset, -7.0 V; interoctapole lens voltage, -22.0 V; ion trap DC offset, -10 V; multiplier voltage, 0.0 V; micro-scan injection time, 200 msec. Segment 3 was split into 3 separate scan events. Scan event 1 collected the  $[M+H]^+$  parent ion at  $m/z$  264.1. Scan event 2 performed MS/MS on this parent ion, using a collision energy of 36%, and collected the daughter ion at  $m/z$  218.1. Scan event 3 performed MS/MS/MS on the daughter ion at  $m/z$  218.1, using a collision energy of 48%, and collected the granddaughter ions at  $m/z$  146.1, 191.1 and 202.1.

For this analysis, the HPLC was operated in an isocratic mode with a flow rate of 1 mL/min. The mobile phase composition was 80:20, methanol:50 mM formate buffer mixture. The sample injection volume was 10  $\mu$ L. The HPLC column was routinely equilibrated for 2 hours prior to use. Following use, the column was washed and stored in 50:50, methanol:H<sub>2</sub>O.

### Specimen Preparation and Extraction

Calibration curves were prepared by serial dilution utilizing human certified negative urine as the sample matrix. Controls were prepared using separate drug standards from those used to create the calibration curve. Calibrators, controls and postmortem urine specimens were prepared and extracted in the following manner.

To facilitate the excretion of 5-HTOL from the body, this compound is extensively conjugated *in vivo* in humans as a glucuronide adduct. Therefore, an initial hydrolysis step was performed on all specimens. Three mL aliquots of calibrators, controls and specimens were individually transferred to 15 mL screw-topped culture tubes. To each sample, 1.00 mL of a 1000 ng/mL (4.50  $\mu$ M) internal standard solution was added.  $\beta$ -Glucuronidase solution (7500 units) followed by 1.00 mL of 0.1 M pH 5.0 sodium acetate buffer was added to each calibrator, control and specimen aliquot. The samples were vortexed briefly and incubated at 70°C for 45 min. Following hydrolysis, samples were allowed to cool to room temperature, then 3.00 mL of a 0.100 M sodium acetate buffer, pH 6.00 and 0.50 mL of a saturated sodium chloride solution were added to each sample, and the tubes were briefly vortexed. Ethyl acetate (9.00 mL) was added to each tube, and the tube was tightly capped. The mixture was then

placed on a rotary mixer and gently mixed for 20 min. Following mixing, the samples were centrifuged at 780xg for 5 min. The organic (upper) layer of each sample was transferred using a clean, disposable pipette to a clean 10 mL conical tube and dried in a water bath at 40°C under a constant stream of nitrogen. Once dryness was achieved, the samples were removed from the evaporator, and both ethyl acetate (50  $\mu$ L) and BSTFA/1%-TMCS (50  $\mu$ L) were added to each. The tubes were capped, vortexed briefly and placed in a heating block at 80°C for 20 min. Following derivitization, the tubes were removed from the heating block, allowed to cool to room temperature and subsequently evaporated to dryness in a water bath at 40°C under a constant stream of nitrogen. The samples were subsequently reconstituted in 50  $\mu$ L of methanol, vortexed briefly and transferred to vials for LC/MS analysis.

### Extraction Efficiency

The recovery of each analyte was determined using the following procedure.<sup>30</sup> Two groups of negative urine samples, X and Y, were extracted and prepared in the same manner as described above, including the addition of internal standard. Group X was spiked with a known concentration of 5-HTOL and 5-HIAA prior to extraction, and group Y was spiked with the same solution following the liquid/liquid extraction step. Upon analysis, the average response factor obtained from group X was divided by the average response factor obtained from group Y to yield the percent recovery value ( $100 \times X/Y = \% \text{ recovery}$ ) for each of the compounds.

### Postmortem Urine Samples

Postmortem urine specimens received by our laboratory are stored at -20°C. Specimens are retained in our storage facility for up to 5 years following collection. The Toxicology and Accident Research Laboratory database was used to identify both positive and negative ethanol urine samples for this study. Our laboratory administratively defines an ethanol negative specimen as any sample containing ethanol at a concentration <10 mg/dL. This 10 mg/dL cutoff is also required by the College of American Pathologists (CAP) in their "whole blood alcohol/volatiles survey" and for CAP laboratory accreditation.

## RESULTS AND DISCUSSION

### Method Validation

The procedure described in this paper provides a rapid, accurate and reproducible method for the simultaneous extraction and quantitation of 5-HTOL and 5-HIAA. A liquid-liquid extraction procedure in combination with

HPLC/APCI-PCI-MS provides both superior separation of these two compounds and detection limits that are well below concentrations expected in most biological specimens.

Derivatization of 5-HTOL and 5-HIAA with TMS was found to be essential for this method. Derivatization of these compounds achieved two necessary objectives. First, the underivatized compounds provided poor chromatographic resolution under mobile phase conditions amenable to LC/MS. And, optimizing the mobile phase to increase APCI-PCI ionization of one compound dramatically decreased the ionization efficiency for the other. This was not unexpected since one compound is an acid and the other a primary alcohol. By producing a TMS derivative of each compound, complete chromatographic resolution was achieved, and the undesirable effects of mobile phase on APCI-PCI ionization was eliminated. Secondly, derivatization increased the mass of the compounds, which practically allowed for MS/MS and MS/MS/MS and enhanced the associated detection limits by approximately 1000 times compared with the underivatized forms.

The internal standard chosen for these experiments was 5-methoxy-2-methyl-3-indoleacetic acid (5-MMIA). This compound is structurally related to both analytes and has similar chromatographic characteristics. Furthermore, it is not endogenous in biological samples of any type. 5-MMIA was found to be stable in solution for an extended period of time.

An ion trap mass spectrometer is a collection device, which allows for the "trapping" or isolation of ions from a target compound, followed by the subsequent formation of unique spectra from these ions. APCI is a soft ionization technique and, when used in the PCI mode, becomes an excellent source of  $[M+H]^+$  parent ions. This ionization technique, in combination with an ion trap, enabled us to perform MS/MS/MS on the 5-HTOL and 5-HIAA derivatives, and MS/MS on 5-MMIA. 5-HTOL had a  $[M+H]^+$  parent ion at  $m/z$  250.1. The parent ion was collected by the ion trap and subjected to MS/MS, resulting in a daughter ion at  $m/z$  232.1. Collecting  $m/z$  232.1 and performing MS on it resulted predominantly in the granddaughter ion at  $m/z$  216.1. The same process was used on 5-HIAA, which had a parent  $[M+H]^+$  ion at  $m/z$  264.1, a daughter ion at  $m/z$  218.1 and several granddaughter ions with high-abundance, including  $m/z$  146.1, 191.1 and 202.1. The internal standard had a  $[M+H]^+$  at  $m/z$  220.1, which, when subjected to MS/MS, resulted in a daughter ion at  $m/z$  174.1. Since it is unlikely that a specimen matrix will produce the same product ions as a target compound, full scan MS/MS and MS/MS/MS spectra for 5-HTOL and 5-HIAA, and MS/MS for

5-MMIA provided the "fingerprints" used for analyte identification and confirmation. The full scan MS/MS and MS/MS/MS spectra are shown in Figures 2-6.

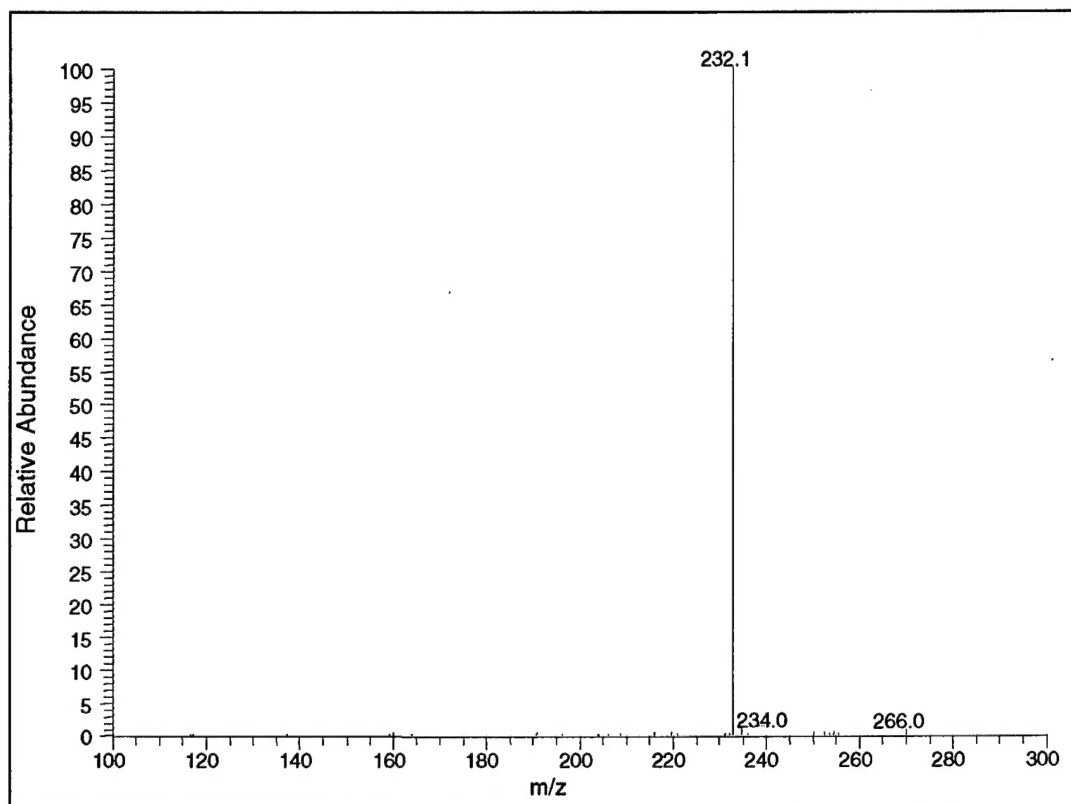
Quantitation of 5-HTOL and 5-HIAA was achieved via an internal standard calibration procedure. Calibration curves were prepared by plotting a linear regression of the analyte/internal standard response factor versus the analyte concentration. Response factors for both compounds were determined for each specimen. The response factor was calculated by dividing the area of the analyte peak by the area of the internal standard peak. The MS/MS/MS ion, at  $m/z$  216.1 was used for 5-HTOL quantitation. Quantitation of 5-HIAA was accomplished using the MS/MS ion at  $m/z$  218.1. The MS/MS ion at  $m/z$  174.1 was used for 5-MMIA.

As can be seen in Figure 7, 5-MMIA, 5-HTOL and 5-HIAA are completely resolved chromatographically. The peak shape is excellent for all three compounds, and the noise was found to be negligible for most injections throughout the analysis. These compounds experienced no interference from endogenous sample matrix components. Typical retention times were 1.59, 2.34 and 3.30 min for 5-MMIA, 5-HTOL and 5-HIAA, respectively. The number of theoretical plates calculated for each compound ranged from 2000 to 5000.

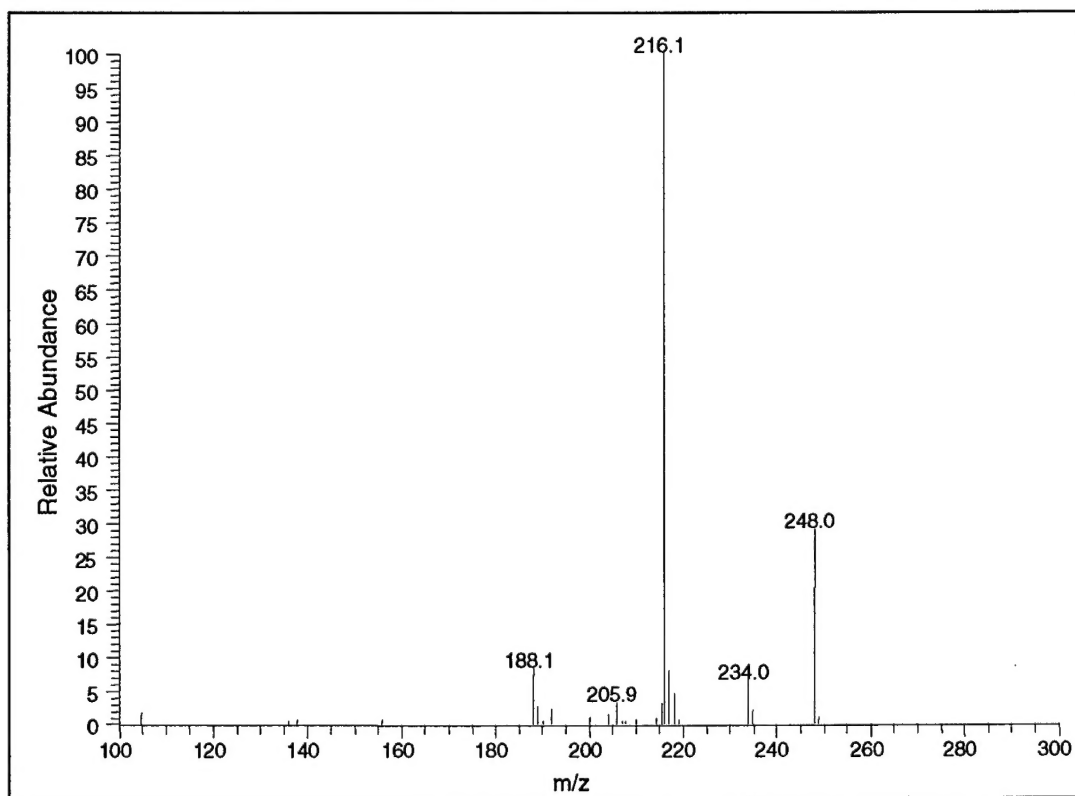
The extraction efficiency of 5-HTOL and 5-HIAA from postmortem urine samples, as described in the experimental section, was determined at 1, 10, 50 and 800 ng/mL. The individual recovery values are presented in Table 1. The recovery of 5-HTOL and 5-HIAA across this broad concentration range averaged 82% and 80%, respectively. These values exceeded our initial expectations, considering the simplicity of the extraction.

The limit of detection (LOD), limit of quantitation (LOQ) and linear dynamic range (LDR) for each analyte are listed in Table 2. The LOD was defined as the lowest concentration of analyte having a minimum signal-to-noise ratio (S/N) of 5, in addition to meeting a MS/MS and MS/MS/MS spectral "fingerprint" confirmation and  $\pm 5\%$  retention time criteria. The LOQ was defined as meeting all LOD criteria plus having a S/N of 10 and having an experimentally determined value within  $\pm 20\%$  of its prepared concentration. The LOD was found to be 0.100 ng/mL for both 5-HTOL and 5-HIAA. The LOQ was found to be 0.390 ng/mL and 0.780 ng/mL for 5-HTOL and 5-HIAA, respectively. The LDR for these two compounds was 0.390 - 800 ng/mL for 5-HTOL and 0.780 - 12800 ng/mL for 5-HIAA. The correlation coefficients for both of the LDR curves exceeded 0.99. Non-linearity was observed with 5-HTOL at concentrations greater than 800 ng/mL, while concentrations above 12,800 ng/mL for 5-HIAA were not evaluated.

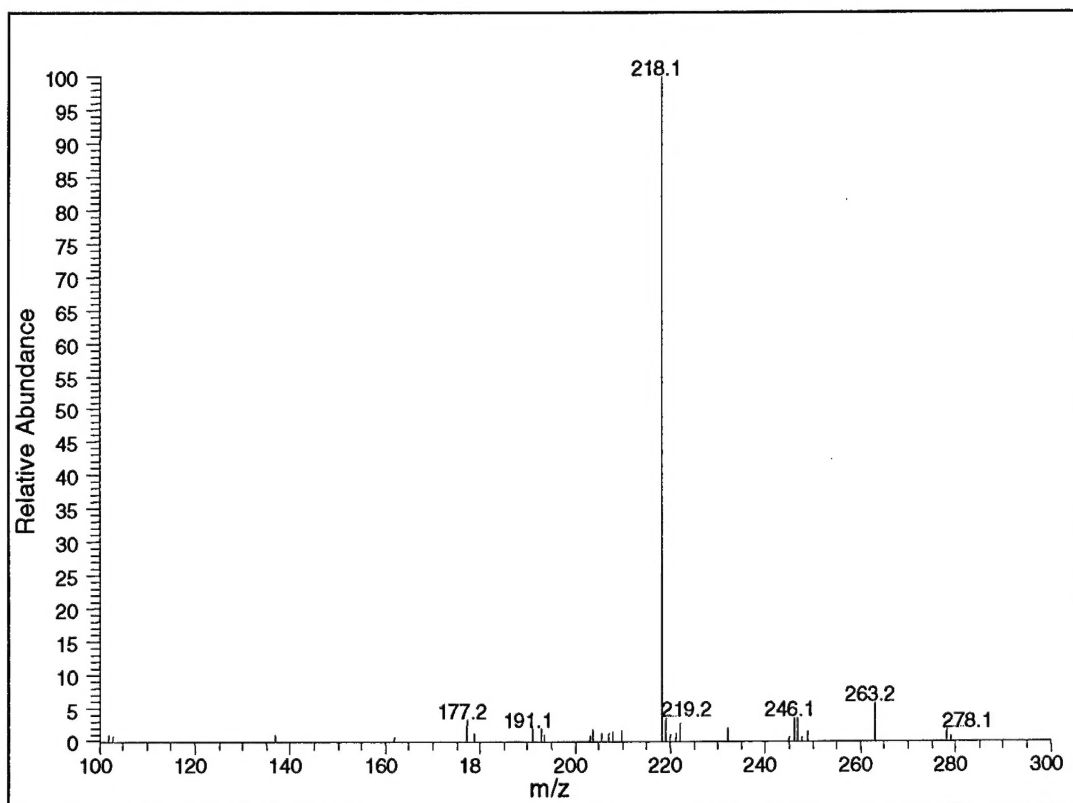




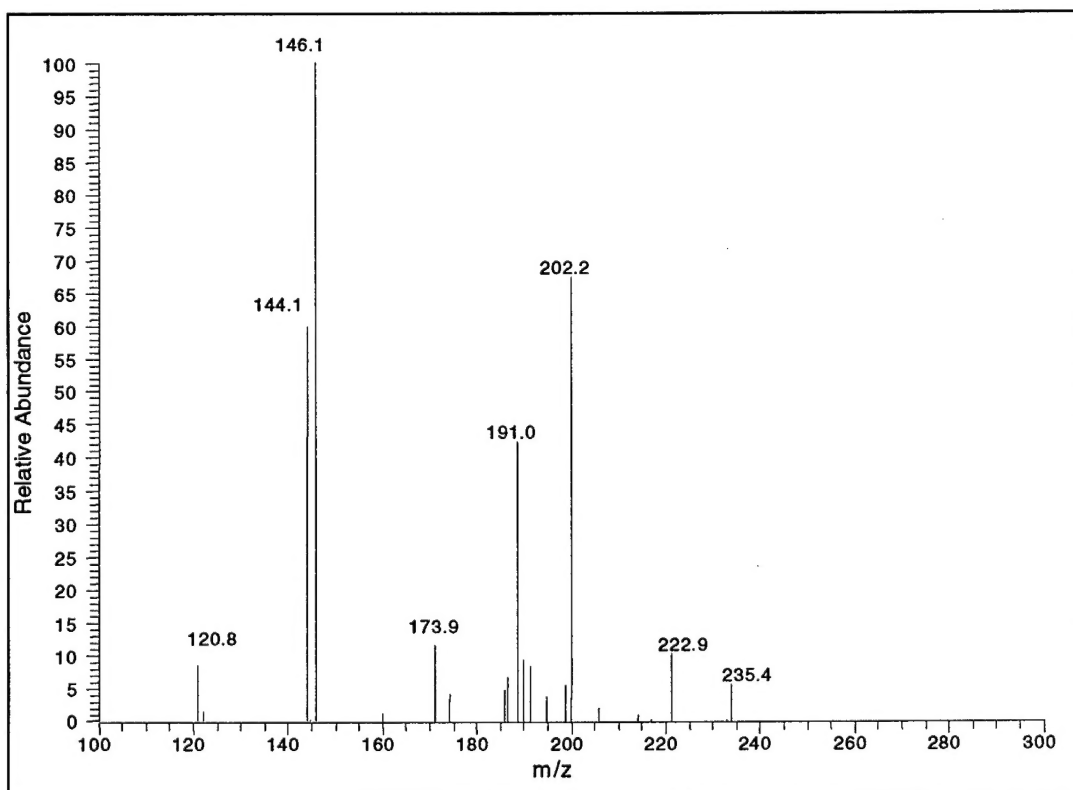
**Figure 2.** MS/MS spectrum of 5-HTOL ( $m/z$  250.1  $\rightarrow$  spectrum).



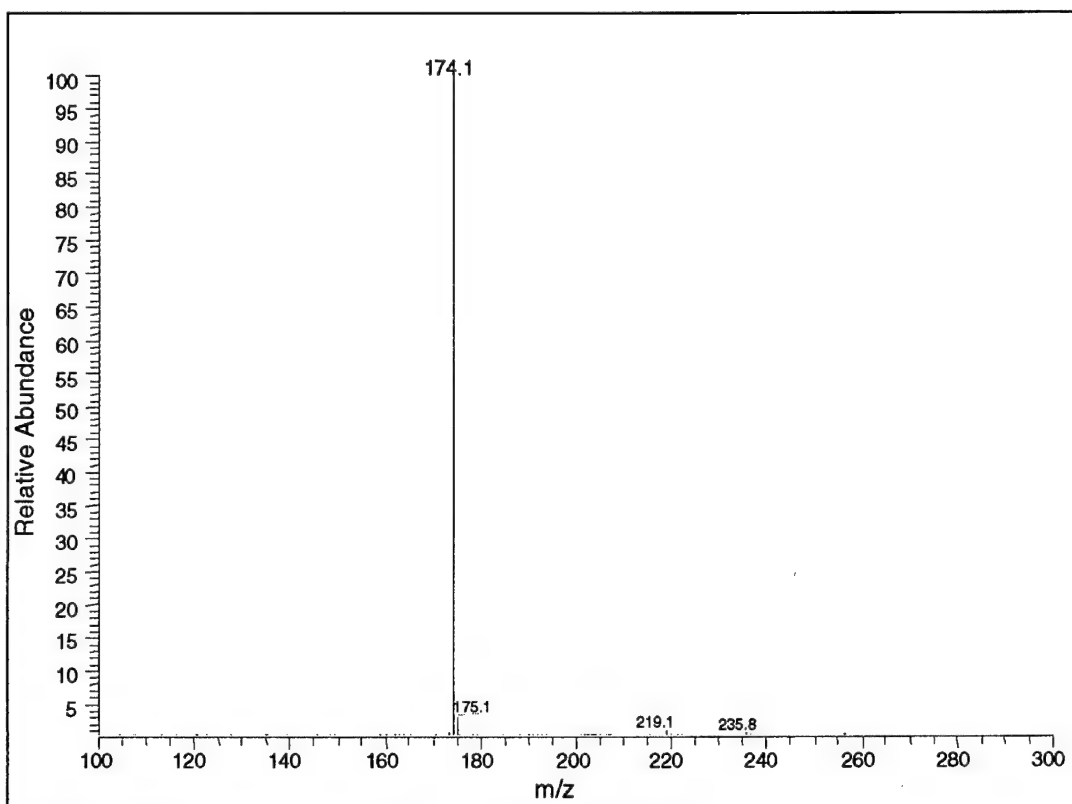
**Figure 3.** MS/MS/MS spectrum of 5-HTOL ( $m/z$  250.1  $\rightarrow m/z$  232.1  $\rightarrow$  spectrum).



**Figure 4.** MS/MS spectrum of 5-HIAA ( $m/z$  264.1  $\rightarrow$  spectrum).



**Figure 5.** MS/MS/MS spectrum of 5-HIAA ( $m/z$  264.1  $\rightarrow$   $m/z$  218.1  $\rightarrow$  spectrum).



**Figure 6.** MS/MS spectrum of 5-MMIA ( $m/z$  220.1  $\rightarrow$  spectrum).

**Table 1.** Percent Recovery  $\pm$  RSD for 5-HTOL & 5-HIAA.\*

Compound	1 ng/mL	10 ng/mL	50 ng/mL	800 ng/mL
5-HTOL	77 $\pm$ 10	81 $\pm$ 7	82 $\pm$ 4	89 $\pm$ 8
5-HIAA	83 $\pm$ 6	89 $\pm$ 2	87 $\pm$ 3	59 $\pm$ 2

\* n=5 for all determinations.

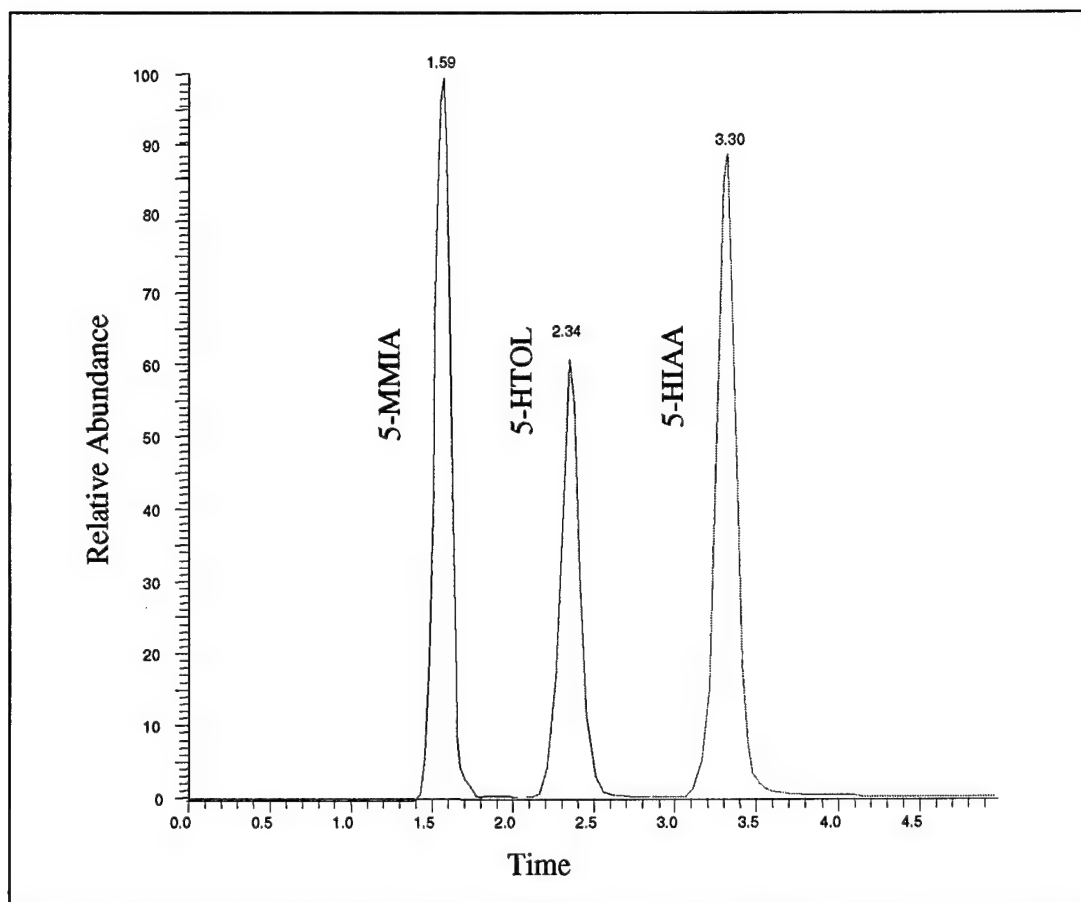
**Table 2.** LOD, LOQ and LDR for 5-HTOL & 5-HIAA.

Compound	LOD (ng/mL)	LOQ (ng/mL)	LDR (ng/mL)
5-HTOL	0.10	0.39	0.39-800
5-HIAA	0.10	0.78	0.78-12800

**Table 3.** Intra-day Accuracy and Precision.\*

	5-HTOL		5-HIAA	
Target Conc. (ng/mL)	1	10	1	10
Mean $\pm$ SD (ng/mL)	1.09 $\pm$ 0.02	9.6 $\pm$ 0.8	1.00 $\pm$ 0.08	9.2 $\pm$ 0.2
Relative Error	+9%	-4%	0%	-8%
R.S.D.	2%	8%	8%	2%

\*n=5 at each concentration, controls were run on day 1.



**Figure 7.** Representative chromatogram of 5-MMIA, 5-HTOL and 5-HIAA. Chromatographic peaks represent ions monitored in SIM mode for each compound as follows: 5-MMIA MS/MS ion at  $m/z$  174.1; 5-HTOL MS/MS/MS ion at  $m/z$  216.1; 5-HIAA MS/MS ion at  $m/z$  218.1. Peaks obtained from a 10  $\mu$ L injection of a standard with an original concentration of 10  $\mu$ g/mL.

Carryover from one sample to the next was not found to be a problem. It was, however, monitored by the use of solvent injections. A methanol blank injected following the highest calibrator showed no carryover contamination. Blanks were also used randomly throughout the sample sequence to verify that no sample-to-sample contamination occurred.

Intra-day (within day) and inter-day (between days) accuracy and precision were examined for this extraction. The accuracy was measured as the relative error between the experimentally determined and prepared concentrations of a sample. The precision was measured as the relative standard deviation (RSD) in the experimentally determined concentrations. Urine controls at 1 and 10 ng/mL were prepared in pools on day 1 and stored in the refrigerator at 4°C until extracted. A calibration curve was extracted along with 5 replicates of each control concentration on day 1 of the experiment. The intra-day relative error and RSD for 5-HTOL were +9% and 2% at 1 ng/mL and -4% and 8% at 10 ng/mL, respectively.

The intra-day relative error and RSD for 5-HIAA were 0% and 8% at 1 ng/mL and -8% and 2% at 10 ng/mL, respectively. These data are summarized in Table 3. Inter-day accuracy and precision were evaluated by extracting 5 replicates of each of two control concentrations on days 2, 4 and 8 and basing the quantitation on the original calibration curve. The inter-day relative error and RSD for 5-HTOL were +1% and 6% at 1 ng/mL and -9% and 4% at 10 ng/mL, respectively. The inter-day relative error and RSD for 5-HIAA were -3% and 6% at 1 ng/mL and -13% and 2% at 10 ng/mL, respectively. The inter-day accuracy and precision are shown in Table 4. The short-term stability of these two compounds at 4°C was evaluated by looking at the data from day 8 alone, as shown in Table 5. After storage for 8 days, the 1 ng/mL control was found to have a 5-HTOL concentration of  $0.99 \pm 0.04$  ng/mL and a 5-HIAA concentration of  $0.91 \pm 0.07$  ng/mL. The 10 ng/mL control was found to have a 5-HTOL concentration of  $8.7 \pm 0.1$  ng/mL and a 5-HIAA concentration of  $8.3 \pm 0.1$  ng/mL. The decrease in

**Table 4.** Inter-day Accuracy and Precision.\*

	5-HTOL		5-HIAA	
Target Conc. (ng/mL)	1	10	1	10
Mean $\pm$ SD (ng/mL)	1.01 $\pm$ 0.06	9.1 $\pm$ 0.4	0.97 $\pm$ 0.06	8.7 $\pm$ 0.2
Relative Error	+1%	-9%	-3%	-13%
R.S.D.	6%	4%	6%	2%

\*n=20, five controls at each concentration were run on days 1, 2, 4 and 8.

**Table 5.** Short Term Stability, 8 Days at 4°C.\*

	5-HTOL		5-HIAA	
Target Conc. (ng/mL)	1	10	1	10
Mean $\pm$ SD (ng/mL)	0.99 $\pm$ 0.04	8.7 $\pm$ 0.1	0.91 $\pm$ 0.07	8.3 $\pm$ 0.1
Relative Error	-1%	-13%	-9%	-17%
R.S.D.	4%	1%	8%	1%

\*n=5 for all determinations.

concentrations at 10 ng/mL was not unexpected, however, due to the ease of autoxidation of these compounds. This decrease may be explained by the use of the original calibration curve for the quantitation of controls extracted up to 8 days later. These results were found to be acceptable for general use and agree well with previously reported short-term stability studies.<sup>27</sup> As a good laboratory practice and in an effort to maintain a high degree of accuracy, we would recommend preparing new calibration curves at the beginning of each new analysis.

Since actual postmortem urine samples are stored for extended periods of time at -20°C, and not 4°C, the long-term stability of 5-HTOL and 5-HIAA at -20°C was investigated. Controls were separated into 4 mL aliquots and stored at -20°C. Five control replicates were then analyzed on days 1, 14, 30, 60, 90 and 180. Fresh calibration curves were prepared and analyzed on each day of analysis. There was no statistical decrease in concentration for any of the controls for all times investigated. Therefore, the degradation of 5-HTOL and 5-HIAA over time in properly stored specimens should not be of significant concern.

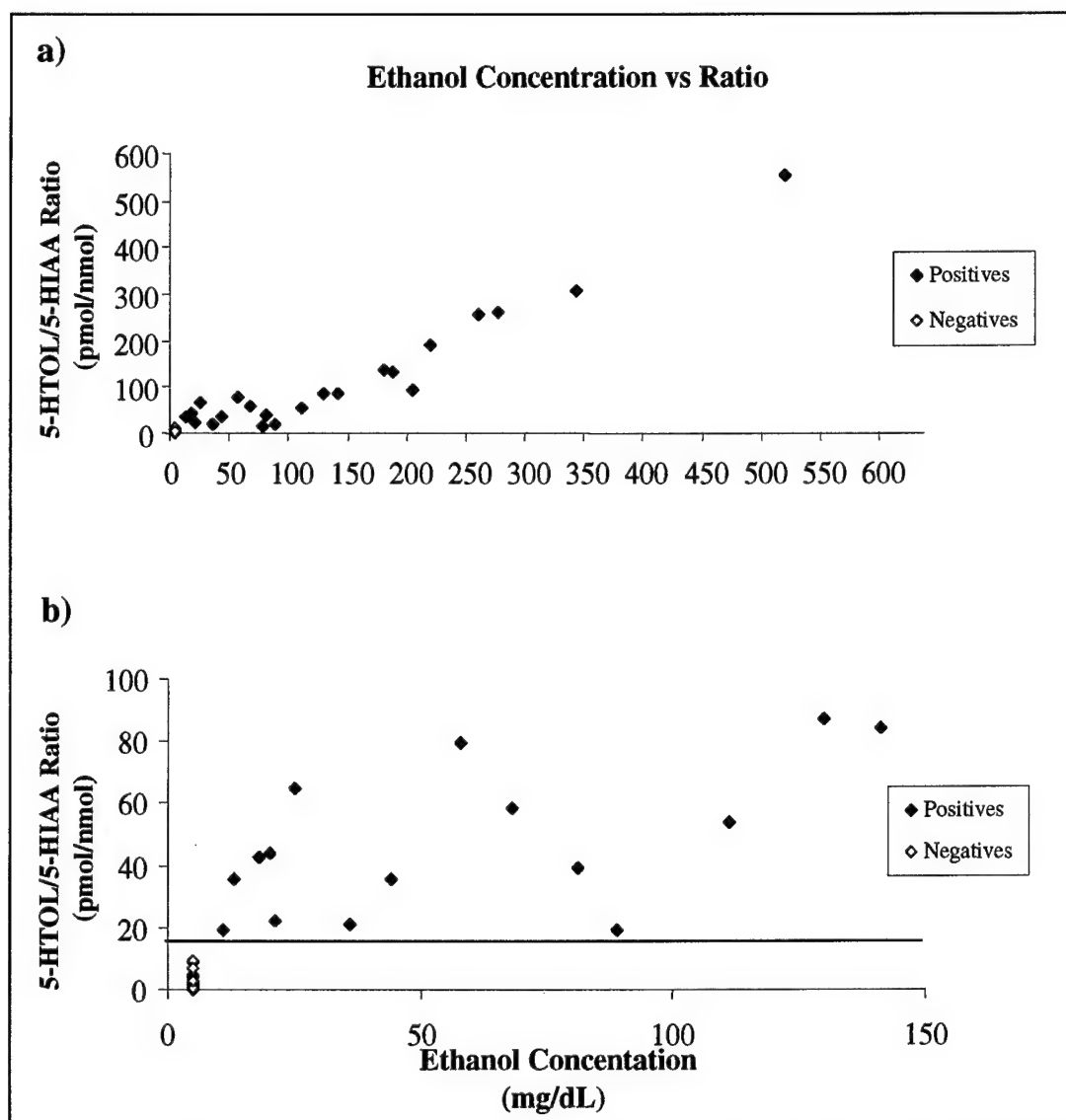
### Forensic Urine Analysis

Several studies have been conducted on live human subjects examining the shift in the urinary 5-HTOL/5-HIAA ratio following consumption of ethanol.<sup>20, 23, 24, 27, 31</sup> These reports have clearly demonstrated elevated 5-HTOL/5-HIAA ratios above those of urine baseline levels following ethanol ingestion. They also found that the more ethanol an individual consumed, the higher the 5-HTOL/5-HIAA ratios were. Furthermore, 5-HTOL/5-

HIAA ratios remained elevated above baseline levels for hours after ethanol could no longer be detected in the body. In one study, both men and women were dosed with 0.8 g/kg ethanol, resulting in an average peak urinary alcohol concentration of 87.4 mg/dL.<sup>20</sup> They found that while ethanol could no longer be detected in the body after 10 hours, the 5-HTOL/5-HIAA ratio remained significantly elevated for up to 16 hours. In a separate study, Hagan *et al.* dosed subjects at 0.6 g/kg, and found that the 5-HTOL/5-HIAA ratio remained elevated for approximately 7 hours after ethanol was eliminated from the body.<sup>24</sup> Helander *et al.* have established a cutoff value for the 5-HTOL/5-HIAA ratio of 15 pmol/nmol, below which ethanol has not been recently consumed.<sup>32, 33, 34</sup> Helander *et al.* also reported a cursory examination of postmortem urine samples with respect to their 5-HTOL/5-HIAA ratio and, while finding substantial individual variations, demonstrated a good correlation between ethanol levels and 5-HTOL/5-HIAA ratios.<sup>23</sup>

We examined 44 urine specimens obtained from fatal aviation accident victims. Of the 44 specimens analyzed, 21 specimens were previously reported negative for ethanol by our laboratory, while 23 specimens were previously reported positive (ethanol > 10 mg/dL). The initial focus of this study was to determine if the previously established 15 pmol/nmol cutoff was appropriate for postmortem urine samples.

The data collected from all 44 specimens are illustrated in Figure 8a. The results show a general trend of increasing 5-HTOL/5-HIAA ratio with increasing urine ethanol concentration. There is, however, substantial inter-individual variation in 5-HTOL/5-HIAA ratios at



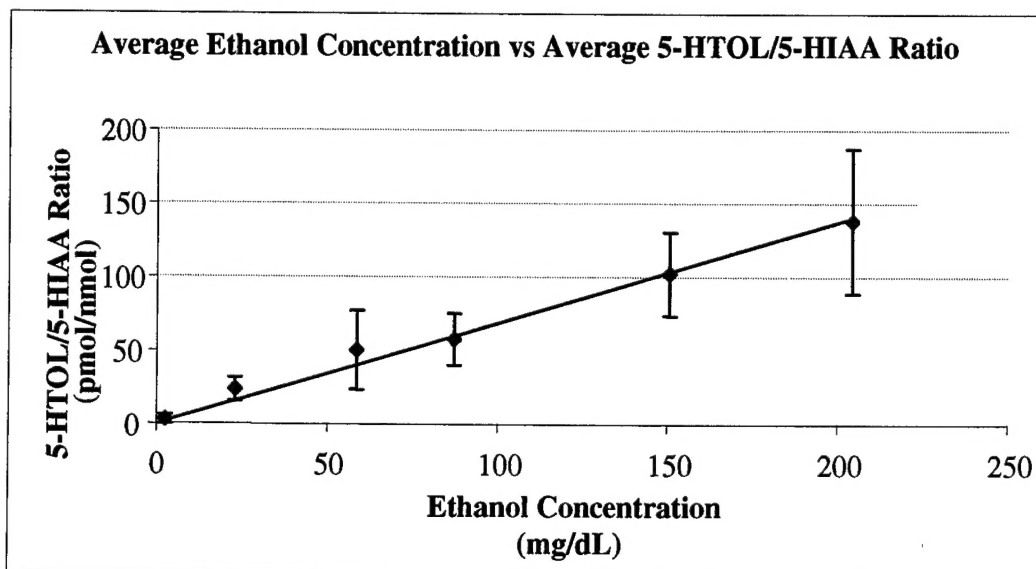
**Figure 8.** Postmortem ethanol concentrations vs 5-HTOL/5-HIAA ratios. "a" shows all postmortem urine data points collected. "b" represents the data for specimens < 150 mg/dL and demonstrates the suitability of the 15 pmol/nmol cutoff.

ethanol concentrations below approximately 150 mg/dL. This area has been expanded and can be seen in Figure 8b. These variations are not unexpected since the specimens utilized in this study are from postmortem sources with unknown dietary histories and relatively unknown medical and alcohol consumption histories. These results may be better represented by averaging groups of individual data points and creating a linear plot of 5-HTOL/5-HIAA ratio vs. ethanol concentration. This approach is illustrated in Figure 9 and clearly demonstrates a relationship between 5-HTOL/5-HIAA ratio and the amount of ethanol detected in a specimen.

5-HTOL/5-HIAA ratios for the 21 ethanol-negative postmortem urine specimens ranged from 0.010 - 9.38

pmol/nmol, with an average of  $2.52 \pm 2.94$  pmol/nmol (mean  $\pm$  S.D.). 5-HTOL concentrations in these specimens ranged from 5.22 nM - 1.67  $\mu$ M (0.930 - 296 ng/mL) with an average of 220 nM (39.5 ng/mL). 5-HIAA concentrations ranged from 5.92  $\mu$ M - 39.1 mM (1.13 - 117  $\mu$ g/mL) with an average of 142  $\mu$ M (27.3  $\mu$ g/mL). All of the negative specimen ratios fell well below the previously established 15 pmol/nmol cutoff. In fact, our average 5-HTOL/5-HIAA ratio is more than 4 standard deviations below the 15 pmol/nmol cutoff ( $2.52 + (2.94 * 4) = 14.3$  pmol/nmol). Therefore, based on our average and S.D., the probability of having a 5-HTOL/5-HIAA ratio above the previously established 15 pmol/nmol cutoff from a person who has not consumed alcohol is less than 1 in 10,000.



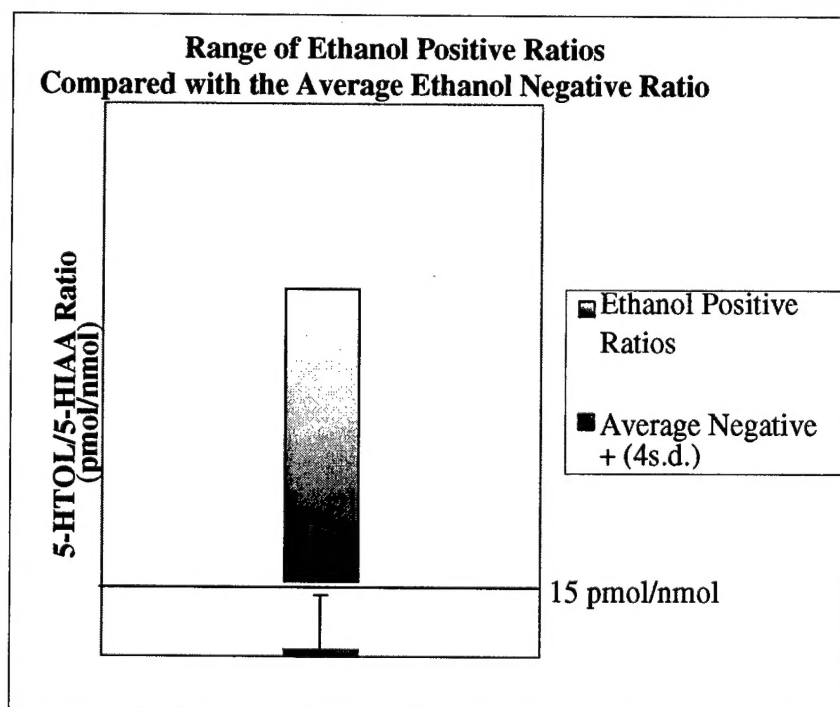


**Figure 9.** Average 5-HTOL/5-HIAA ratio ( $\pm$  s.d.) vs average ethanol concentration.

The 23 ethanol-positive specimens had ethanol concentrations ranging from 11 to 520 mg/dL. The corresponding 5-HTOL/5-HIAA ratios ranged from 19.1 to 551 pmol/nmol. All of the positive specimens ratios fell well above the previously established 15 pmol/nmol cutoff. Figure 10 graphically shows the difference between the 5-HTOL/5-HIAA ratios of ethanol-positive and ethanol-negative specimens. Our data indicate that

the previously established 15 pmol/nmol cutoff is highly appropriate for postmortem urine specimens, and we recommend it be employed as the ethanol positive cutoff ratio in such studies.

We applied this novel LC/MS method to urine samples from 2 cases suspected of experiencing postmortem ethanol formation. Case #1 was obtained from a fatal aviation accident that occurred in remote, mountainous terrain.



**Figure 10.** Average ethanol negative 5-HTOL/5-HIAA ratio (+ 4s.d.) vs range of ethanol positive ratios.

The victim was not recovered from the accident scene for more than 24 hours. This case had a urine ethanol concentration of 31 mg/dL. The case also had a blood ethanol value of 93 mg/dL and a vitreous humor of 3 mg/dL. These specimens were also notably missing other volatiles. The abnormal nature of the ethanol distribution in these 3 biological matrices suggests postmortem ethanol formation. Conversely, the lack of other commonly analyzed volatiles may suggest the absence of postmortem ethanol production.<sup>16, 35, 36, 37, 38, 39, 40, 41</sup> However, the use of other volatiles as a marker for postmortem ethanol formation can be misleading and, therefore, caution must be exercised.<sup>2</sup> To conclusively determine if recent ethanol ingestion occurred, we investigated the 5-HTOL/5-HIAA ratio. We found the 5-HTOL/5-HIAA ratio to be 1.62 pmol/nmol, which was substantially below the established 15 pmol/nmol cutoff. This result indicates an absence of recent ethanol ingestion and supports the conclusion that the ethanol present in this case was due to postmortem formation.

Case #2 was an aviation fatality that was recovered from water approximately 2.5 months after the accident occurred. The urine and blood ethanol concentrations were 31 mg/dL and 16 mg/dL, respectively. Vitreous humor, heart and skeletal muscle were also analyzed and were found to have ethanol concentrations of 17 mg/dL, 8 mg/hg and 12 mg/hg, respectively. Various volatiles, including large amounts of acetaldehyde, n-propanol and n-butanol were also present in these samples. The blood and tissue samples were putrefied and the urine was red and bloody. While the distribution of ethanol between fluid and tissues was reasonably consistent with ethanol consumption, the visual confirmation of sample putrefaction and the presence of various volatiles suggests that the ethanol found in this case may be due to postmortem formation. We found the 5-HTOL/5-HIAA ratio to be 1.95 pmol/nmol. This value is below 15 pmol/nmol, thus indicating the absence of recent ethanol ingestion. The presence of ethanol in this case was reported as postmortem formation.

## CONCLUSION

This study shows the effectiveness of LC/MS in the analysis of 5-HTOL and 5-HIAA. It also demonstrates the utility of the 5-HTOL/5-HIAA ratio in determining ethanol origin in postmortem urine specimens. One of the most important aspects of this novel method is the simultaneous analysis of both compounds using one extraction method and one analytical technique. This

greatly increases the precision in the methodology and, thus, the certainty of subsequent conclusions. The relative simplicity of this procedure should make the 5-HTOL/5-HIAA ratio methodology more readily applicable to the field of forensic toxicology. The application of this easy, rapid and accurate method is not limited to postmortem samples. Contested antemortem ethanol results could also readily be evaluated through the use of this procedure. The postmortem formation of ethanol remains one of the biggest concerns in the forensic toxicology community; however, the utilization of this method, as it applies to postmortem urine samples, may offer a substantial improvement in such investigations.

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